CD40 (Human) ELISA Kit

Catalog Number KA1061
96 assays
Version: 03

Intended for research use only
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**Introduction**

**Intended Use**

For quantitative detection of human CD40 in cell culture supernates, serum, plasma (heparin, EDTA), saliva, urine and human milk.

**Background**

CD40 is a cell surface receptor that is expressed on the surface of all mature B cells, most mature B-cell malignancies, and some early B-cell acute lymphocytic leukemias, but is not expressed on plasma cells.\(^1\) The CD40 gene is localized to the long arm of chromosome 20, bands q12-q13.2.\(^2\) The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.\(^3\)

**Principle of the Assay**

CD40 (Human) ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for CD40 has been precoated onto 96-well plates. Standards and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for CD40 is added subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the human CD40 amount of sample captured in plate.
General Information

Materials Supplied

List of component

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>96-well plate precoated with anti-human CD40 antibody</td>
<td>1</td>
</tr>
<tr>
<td>Lyophilized recombinant human CD40 standard</td>
<td>10 ng/tube x 2</td>
</tr>
<tr>
<td>Biotinylated anti-human CD40 antibody, dilution 1:100</td>
<td>130 µl</td>
</tr>
<tr>
<td>Avidin-Biotin-Peroxidase Complex (ABC), dilution 1:100</td>
<td>130 µl</td>
</tr>
<tr>
<td>Sample diluent buffer</td>
<td>30 ml</td>
</tr>
<tr>
<td>Antibody diluent buffer</td>
<td>12 ml</td>
</tr>
<tr>
<td>ABC diluent buffer</td>
<td>12 ml</td>
</tr>
<tr>
<td>TMB color developing agent</td>
<td>10 ml</td>
</tr>
<tr>
<td>TMB stop solution</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Storage Instruction

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles.

Materials Required but Not Supplied

✓ Microplate reader in standard size.
✓ Automated plate washer.
✓ Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
✓ Clean tubes and Eppendorf tubes.
✓ Washing buffer (neutral PBS or TBS).

Preparation of 0.01M TBS:
Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H₂O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Preparation of 0.01 M PBS:
Add 8.5g sodium chloride, 1.4g Na₂HPO₄ and 0.2g NaH₂PO₄ to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.
Precautions for Use

- To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
- The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
- Before using Kit, spin tubes and bring down all components to bottom of tube.
- Duplicate well assay was recommended for both standard and sample testing.
- Don’t let 96-well plate dry, dry plate will inactivate active components on plate.
- Don’t reuse tips and tubes to avoid cross contamination.
- To avoid to use the reagents from different batches together.
- In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37°C for 30 min before using.
Assay Protocol

Reagent Preparation

✓ Reconstitution of the human CD40 standard: CD40 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of CD40 standard (10ng per tube) are included in each kit. Use one tube for each experiment.
  • 10,000 pg/ml of human CD40 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
  • 1000 pg/ml of human CD40 standard solution: Add 0.1 ml of the above 10 ng/ml CD40 standard solution into 0.9 ml sample diluent buffer and mix thoroughly.
  • 500 pg/ml → 15.6 pg/ml of human CD40 standard solutions: Label 6 Eppendorf tubes with 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 1000 pg/ml CD40 standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 10 ng/ml standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

✓ Preparation of biotinylated anti-human CD40 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
  • The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
  • Biotinylated anti-Human CD40 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human CD40 antibody to 99µl antibody diluent buffer.)

✓ Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
  • The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
  • Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluents buffer.)

Sample Preparation

✓ Sample Preparation and Storage
Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
- Cell culture supernate: Remove particulates by centrifugation, analyze immediately or aliquot and store sample at -20°C.
- Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 x g for 15 min. Analyze the serum immediately or aliquot and store samples at -20°C.
- Plasma: Collect plasma using heparin, EDTA as an anticoagulant. Centrifuge for 15 min at 1500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20°C.
- Saliva: Collect saliva using a collection device without any protein binding or filtering capabilities such as a Salivette or aliquot and store samples at -20°C.
- Urine: Aseptically collect the first urine of the day, micturate directly into a sterile container. Remove particular impurities by centrifugation, assay immediately or aliquot and store samples at -20°C.
- Human milk: Centrifuge for 15 min at 1500 x g at 2-8°C. Collect the aqueous fraction and repeat this process 3 times. Filter through a 0.2µm filter and assay immediately or aliquot and store samples at -80°C.

Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. The sample must be well mixed with the diluents buffer.

- High target protein concentration (10-100 ng/ml). The working dilution is 1:100. i.e. Add 1 µl sample into 99 µl sample diluent buffer.
- Medium target protein concentration (1-10 ng/ml). The working dilution is 1:10. i.e. Add 10 µl sample into 90 µl sample diluent buffer.
- Low target protein concentration (15.6-1000 pg/ml). The working dilution is 1:2. i.e. Add 50 µl sample to 50 µl sample diluent buffer.
- Very Low target protein concentration (≤15.6 pg/ml). No dilution necessary, or the working dilution is 1:2.

Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard CD40 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of CD40 amount in samples.

1. Aliquot 0.1 ml per well of the 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml human CD40 standard solutions into the precoated 96-well plate. Add 0.1 ml of the sample diluent buffer into the control well (Zero well). Add 0.1 ml of each properly diluted sample of human cell culture supernates, serum, plasma (heparin, EDTA), saliva, urine or human milk to each empty well. See
Sample Dilution Guideline above for details. It is recommended that each human CD40 standard solution and each sample is measured in duplicate.

2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1 ml of biotinylated anti-human CD40 antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash the plate 3 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (Plate Washing Method: Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1 ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
8. Add 90 μl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 25-30 min (Note: For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human CD40 standard solutions; the other wells show no obvious color).
9. Add 0.1 ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

Summary
1. Add samples and standards and incubate the plate at 37°C for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37°C for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37°C in dark for 25-30 min.
5. Add TMB stop solution and read.
Data Analysis

Calculation of Results

For calculation, \(( \text{O.D.}_{450} \text{ of each well}) - (\text{O.D.}_{450} \text{ of Zero well}) \). The standard curve can be plotted as the relative \(\text{O.D.}_{450}\) of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human CD40 concentration of the samples can be interpolated from the standard curve.

*Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.*

✓ Typical result

Typical Data Obtained from Human CD40

<table>
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<th>Concentration (pg/ml)</th>
<th>0</th>
<th>15.6</th>
<th>31.2</th>
<th>62.5</th>
<th>125</th>
<th>250</th>
<th>500</th>
<th>1000</th>
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<td>O.D.</td>
<td>0.010</td>
<td>0.077</td>
<td>0.147</td>
<td>0.221</td>
<td>0.419</td>
<td>0.997</td>
<td>1.620</td>
<td>2.394</td>
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(TMB reaction incubate at 37°C for 25 min)

This standard curve was generated for demonstration purpose only. A standard curve must be run with each assay.

Performance Characteristics

✓ Range: 15.6 pg/ml-1000 pg/ml
✓ Sensitivity: < 1 pg/ml
✓ Specificity: Natural and recombinant human CD40
✓ Cross-reactivity: No detectable cross-reactivity with other relevant proteins.
Resources

References

3. "Entrez Gene: CD40 CD40 molecule, TNF receptor superfamily member 5".
### Plate Layout

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